Production and Characterization of Monoclonal Antibodies Against the Lethal Factor Component of *Bacillus anthracis* Lethal Toxin

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Running title: Monoclonal Antibodies to Anthrax Lethal Factor

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST THE LETHAL FACTOR COMPONENT OF BACILLUS ANTHRACIS LETHAL TOXIN	5. TYPE OF REPORT & PERIOD COVERED: Publication / 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Stephen F. Little; Stephen H. Leppla - NIDR, NIH, Bethesda, MD; Arthur M. Friedlander	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS: U.S. Army Medical Research Institute of Infectious Diseases, Bacteriology Division, SGRD-UIB, Fort Detrick, Maryland 21701-5011	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102A 3M161102BS12 AB 002 APC# MEDA
11. CONTROLLING OFFICE NAME AND ADDRESS: U.S. Army Medical Research and Development Command, Fort Detrick, Frederick, Maryland 21701-5012	12. REPORT DATE: 29 November 1989 / 13. NUMBER OF PAGES: 38
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report): Unclassified / 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE: N/A

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Submitted to Infection and Immunity for consideration of publication

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Monoclonal antibodies
Anthrax lethal toxin
Bacillus anthracis
Lethal factor

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The lethal toxin of Bacillus anthracis consists of two components, protective antigen and lethal factor. Protective antigen is cleaved after binding to cell receptors, yielding a receptor-bound fragment that binds lethal factor. Sixty-one monoclonal antibodies to the lethal factor protein have been characterized for specificity, antibody subtype, and ability to neutralize lethal toxin. Three monoclonal antibodies (10G3, 2E7 and 3F6) neutralized lethal toxin in Fisher 344 rats. However, in a macrophage cytolysis assay, monoclonal anti-10G3, 2E7, 10G4, 10D4, 13D10, and 1D8, but not 3F6, were found to neutralize

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. Abstract (continued)

lethal toxin. Binding studies showed that five of the monoclonal antibodies that neutralized lethal toxin in the macrophage assay (10G3, 2E7, 10G4, 10D4, and 13D10) did so by inhibiting the binding of lethal factor to the protective antigen fragment bound to cells. Monoclonal antibody 1D8, which was also able to neutralize lethal toxin activity after LF was prebound to cell-bound PA, only partially inhibited binding of lethal factor to protective antigen. Monoclonal antibody 3F6 did not inhibit the binding of lethal factor to protective antigen. A competition-binding ELISA showed that at least four different antigenic regions on lethal factor were recognized by these seven neutralizing hybridomas. The anomalous behavior of 3F6 suggests that it may induce a conformational change in lethal factor. Differences in neutralizing activity of monoclonal antibodies were related to their relative affinity, epitope specificity, and the type of assay.

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Collectively, three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF) comprise the two exotoxins of Bacillus anthracis, lethal toxin and edema toxin. In several respects, the toxin components interact to fit the pattern of A-B type protein toxins (12). Individually, these three proteins have no known toxic activity in animals. However, when PA is combined with LF, lethal toxin is formed, and combination of PA with EF forms edema toxin. PA (83 kDa) is cleaved by a cellular protease after binding to a cell receptor (18,26). The larger, C-terminal, 63.5-kDa fragment (PA63) remains bound to the cell receptor. Subsequent binding of either LF or EF leads to expression of either lethal or edema toxin activity, respectively. EF has been shown to be a calcium- and calmodulin-dependent adenylate cyclase (16) and edema toxin produces edema in the skin of animals (2). No enzymatic mechanism of action for LF has been described yet. Lethal toxin causes death in experimental animals (28) and lyses macrophages and macrophage-like cell lines (11,26).

Monoclonal antibodies have been described for PA (19) and have proven useful for confirmation of the model proposed for interaction of anthrax toxin with cells. This report describes the preparation and characterization of hybridoma clones to LF. In addition to utilizing Fisher 344 rats to test the ability of the LF MAbs to neutralize lethal toxin, we exploited the unique sensitivity of certain macrophage cell lines to lethal toxin (26), and adopted a convenient colorimetric assay for cell

viability (13,21) that can be performed in 96-well microtiter plates to evaluate the ability of LF MAbs to neutralize lethal toxin. This in-vitro, cell-based assay allows the testing of individual toxin components in various combinations with MAbs.

MATERIALS AND METHODS

Abbreviations. EF, edema factor; ELISA, enzyme-linked immunosorbent assay; EMEM, Eagle minimum essential medium with Earles balanced salts solution; HBSS, Hank's balanced salt solution; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Ig, immunoglobulin; kDa, kilodaltons; LDH, lactic dehydrogenase; LF, lethal factor; MAb, monoclonal antibody; PA, protective antigen; PA63, 63.5-kDa C-terminal fragment purified from trypsin-cleaved PA.

General methods. Several previously reported procedures (19) were used, some with minor changes. An ELISA-based subtype kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was used to determine antibody subtype and subclass specificity. Monoclonal antibody affinity was calculated using logarithmetically transformed data in a modified linear regression analysis computer program (J. R. Lowe, Fed. Proc. 45:1852, 1986). Immunodiffusion plates, used to measure Ig concentration by radial immunodiffusion, were also prepared with rabbit anti-mouse IgA (Kirkegaard & Perry, Gaithersburg, Md). Immunoglobulin concentrations were calculated from IgA (Meloy Laboratories, Inc., Springfield, Va) standard curves after incubation of plates for 48 h. The monoclonal nature of selected cell lines was identified by isoelectric focusing of purified IgG MAbs. Competition between MAbs for a single antigenic site was measured by an ELISA competitive-binding assay. The percent of biotinylated MAb bound in the presence of competing, unlabeled

MAb was calculated by the following formula: % binding = (test well A_{405}/control well A_{405}) X 100.

Bacillus anthracis antigens. Purified PA, LF, and EF antigens were prepared as previously described (17). PA63 was prepared by cleaving PA with trypsin and separating fragments by chromatography on Mono Q resin (3). Each protein was at least 95%, pure as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Hybridomas. Female BALB/c mice were immunized with purified LF antigen as shown in Table 1. Hybrid cell lines were prepared by fusing spleen cells from immunized mice with SP2/O-Ag14 myeloma cells (8). Hybridoma cultures were screened for reactivity with LF by ELISA (described below) and positive hybridomas were subcloned twice by limiting dilution. Ascites from each hybridoma, produced by injecting approximately $1x10^6$ hybridoma cells i.p. into BALB/c female mice 3 weeks after injection of 0.5 ml i.p. of pristane (2,6,10,14-tetramethyl pentadecane), were pooled, clarified by centrifugation, and aliquots stored at -70°C.

One of the hybridoma clones prepared in this report was previously been used for purification of LF from crude culture supernatant fluids by affinity chromatography (15,20).

ELISA. The ELISA was as previously described (19), except that bound mouse immunoglobulins were detected with a 1:1000 dilution of horseradish peroxidase (HRP) conjugated to goat antibody to mouse immunoglobulins G, M, and A (0.5 mg per ml;

Kirkegaard and Perry). Absorbance was read on a Bio-Tek Autoreader Model EL310 (Bio-Tek Instruments, Inc. Winooski, Vt.) Wells with A_{405} > 0.20 were considered positive.

Purification of Monoclonal Antibody. Immunoglobulins from selected ascitic fluids were purified by FPLC by using a 1.0 ml Mono S cation exchange chromatography column (Pharmacia-LKB, Piscataway, N.J.). Ascitic fluids were diluted 1:2 in buffer A [50 mM acetate buffer (pH 5.0)], centrifuged (10,500 x g, 5 min) in a Beckman Microfuge 12 (Beckman Instruments, Fullerton, Calif.), and filtered through a 0.22 μm filter before 1 ml volumes were added on the column. Elution was carried out with a 0-40% gradient with buffer B [buffer A + 1 M NaCl (pH 5.0)]. Fractions containing specific antibody were identified by ELISA and concentrated either by precipitation with 50% saturated $(NH_4)_2SO_4$ (pH 7.5), or by ultrafiltration (Centricon 10, Amicon Division W. R. Grace & Co., Danvers, Ma.). Samples were dialyzed extensively against either 0.1 M $NaHCO_3$, 0.15 M NaCl (pH 8.5) buffer, if the sample was to be labeled with biotin (see below) or against 10 mM Tris hydrochloride (pH 8.0). One MAb (10D4) formed a precipitate in the 10 mM Tris hydrochloride buffer (pH 8.0) and was dialyzed instead against 0.01 M sodium phosphate, 0.15 M NaCl (pH 7.3). Samples were frozen at -70°C.

Protein analysis. Protein concentrations were determined by the method of Redinbaugh and Campbell (24). Mouse IgG was used as the protein standard.

Biotinylation of purified MAb. Purified MAbs were

biotinylated by adding 1 mg biotin (Long Arm) NHS (50 mg/ml in dimethyl sulfoxide; Vector Laboratories, Inc., Burlingame, Ca.) per 10 mg protein. Biotin (Long Arm) NHS is an N-hydroxy-succinimide derivative of biotin with an aminchexanoate spacer arm. After incubation for 2 h at room temperature, the biotinylated MAbs were dialyzed against 10 mM Tris hydrochloride (pH 7.5) and frozen at -70°C. For use, glycerol was added to 50%, and vials were held at -20°C.

In-vivo neutralization. Neutralization of lethal toxin was assayed in male Fisher 344 rats (225-250 g) by injecting i.v. 1 ml mixtures of 40 μg PA, 8 μg LF, and ascitic fluid containing 1 mg Ig of each monoclone after preincubation at 37°C for 1 h. Only one rat per monoclone was tested initially. Ascites fluids of MAb that protected the rats were retested at lower concentrations until no protection was observed. Ascites fluids that demonstrated a delayed time to death were retested at a higher concentration (5 mg Ig) to determine if protection could be obtained. Rats were observed for 2 days.

Colorimetric assay for cell viability. An in-vitro colorimetric assay, developed to assess cell growth, survival, and viability (13,21), was modified for use as a neutralization assay. J774A.1 murine macrophage cells, obtained from American Type Culture Collection, Rockville, Md, were cultured in DMEM, 0.45% glucose, 100 Units penicillin per ml, 100 μg streptomycin per ml, and 5% heat-inactivated fetal bovine serum. Cells were scraped from confluent cultures, resuspended to 4-5 x 10^5 cells

per ml in the above medium supplemented with 0.025 M HEPES, plated in 96-well plates (100 µl per well), and incubated overnight at 37°C. Three different neutralization protocols were developed to study interactions between J774A.1 cells, PA, LF, and MAb. First, ascitic fluid containing MAb was diluted in lethal toxin (400 ng per ml PA + 40 ng per ml LF), incubated for 1 h, 37°C, and subsequently added to the J774A.1 cells. All MAbs were tested in this assay. Second, J774A.1 cells were preincubated with either PA or PA63 (400 ng per ml) at 4°C for 2 h. In separate 96-well plates, ascitic fluid containing MAb was diluted in LF (40 ng/ml) and incubated for 1 h, 37°C. After aspirating the PA or PA63 from the cells and washing the wells with 200 µl incubation medium, MAb dilutions in LF were added. Third, J774A.1 cells were sequentially incubated with PA63 (800 ng per ml) and LF (80 ng per ml) at 4°C for 2 h each. Cells were washed after each incubation step with 200 µl incubation medium. Dilutions of ascitic fluids containing MAb in incubation medium were then added. Only selected MAbs were tested by the latter two protocols.

After addition of the MAb dilutions to the cells, the plates were incubated for 3.5-4 h at 37°C. Twenty microliters of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue, Sigma Chemical Co., St. Louis, Mo) at 5 mg per ml in HBSS with 10 mM HEPES was then added to each well, and the plates reincubated. After an overnight incubation, the plates were centrifuged (900 x g, 3 min), contents of each well

aspirated, and 100 µl of acidified isopropanol (9.6 ml isopropanol + 0.4 ml 1 M HCl) was added to each well. After 1 h the contents of each well were mixed thoroughly and A_{540} nm was measured on a Bio-Tek Autoreader Model EL 310. Assays were repeated to determine the end-point titer of those MAbs that neutralized the lethal toxin. The percent neutralization was calculated from the following formula:

$$\% \text{ Neutralization} = 100 \times ((A_{540} \text{ toxin + MAb})/(A_{540} \text{ MAb}))$$

Each calculation was performed by using the average A_{540} of pairs of wells receiving either toxin + MAb or MAb alone. The A_{450} of wells containing only incubation medium averaged about 0.800.

Stimulation of cell growth, even in the presence of toxin, was occasionally observed with MAbs that contained low concentrations of Ig. This activity was rapidly lost upon further dilution and was therefore attributed to the high concentration of serum proteins in the MAb sample and was not considered an ability to neutralize lethal toxin. These results are not shown.

Lactic dehydrogenase assay. The ability of ascitic fluid MAbs to neutralize lethal toxin was also determined by measuring the amounts of LDH present in control or toxin-treated cell monolayers. J774A.1 cells (2-4 x 10^5 in 1 ml) were grown in 24-well tissue culture plates in DMEM, 10% fetal bovine serum, 100 Units penicillin per ml, and 100 µg streptomycin per ml until 80 to 90% confluency. Two-hundred microliters of each monoclonal antibody (500 µg Ig per ml) was incubated with 100 µl LF (250 ng

per ml) for 1 h at 37°C with shaking. Three-hundred fifty microliters PA (250 ng per ml) and 150 µl of the MAb + LF mixture were added to the cells in duplicate wells and incubated for 18 h at 37°C. LDH retained in the cells was assayed as described previously (11). Results were expressed as percent neutralization =

$$100 \times \frac{(\text{LDH with toxin + MAb}) - (\text{LDH with toxin})}{(\text{LDH without toxin or MAb}) - (\text{LDH with toxin})}.$$

The LDH in cells incubated with toxin alone was ≤ 10% of that of control cells not exposed to toxin.

Binding studies with ^{125}I-LF. Binding studies with radioiodinated LF were based upon previously reported studies (3). L6 cells (2-4 x 10^5 in 1 ml; obtained from the American Type Culture Collection), cultured in triplicate 24-well plates, were washed with cold HBSS for 5 min before 500 µl PA (500 ng per ml), diluted in EMEM medium (EMEM without sodium bicarbonate, 1% bovine serum albumin, 20 mM HEPES, 100 Units penicillin per ml, 100 µg streptomycin per ml), was added to each well. The plates were incubated for 18 h at 4°C on ice. In a separate 24-well plate, 200 µl of each ascitic fluid containing MAb (500 µg Ig per ml), 40 ng ^{125}I-LF (specific activity = 1-2 x 10^7 cpm/µg), and EMEM medium to bring the volume of each well to 500 µl, was incubated with shaking for 2 h at 4°C. After this incubation, each well received 500 µl EMEM medium. The L6 cells were washed twice with cold HBSS, and 400 µl of the MAb + ^{125}I-LF mixture was added per well. After incubation for 6 h at 4°C on ice, the

wells were then washed three times with cold HBSS, the cells solubilized with 1 ml 0.1 N NaOH, and radioactivity was counted. The data are reported as percent inhibition of the binding occurring in control wells incubated in the absence of MAb, which was approximately 10,000 cpm/well.

RESULTS

MAb characterization. Sixty-three MAbs to the LF component of the lethal toxin of Bacillus anthracis were obtained from three separate fusions (I, II, and III) by using the SP2/O myeloma cell line and spleen cells from mice immunized with LF antigen. Hybridoma clones specifically mentioned in this report are listed in Table 2 with their subtypes and percent inhibition of binding of ^{125}I-LF to PA. Designations of clones in the text of this report consist of the first set of three alphanumeric characters of the complete identifier. Fifty-eight of the sixty-three MAbs were specific for LF and did not bind to PA or EF, as determined by ELISA. Three MAbs cross-reacted with EF (10B2, 10D4, and 10G4) and two with PA (1F11 and 2G2) (Fig. 1). A detailed analysis of the cross-reactions of these five MAbs will be presented elsewhere. Forty-three of the clones were IgG (28 IgG_1, 13 IgG_{2a}, 2 IgG_3), 18 were IgM clones, and two were IgA clones. Isoelectric focusing analysis, performed on IgG clones of similar isotypes located in closely adjacent wells on the initial fusion plate, demonstrated unique banding patterns and pI differences between all MAbs tested except for two pairs of clones (3E6/3F6 and 10E4/10F4) (data not shown). Isoelectric focusing analysis of mixtures of these two clone pairs demonstrated their apparent identity (data not shown). Although 3E6 and 3F6 probably are identical, they were both carried through subsequent analysis. The results indicate that approximately 61 distinct MAb clones were obtained against LF.

Isoelectric focusing was not performed on the IgM clones.

Protection assays. Neutralization assays demonstrated that four MAbs (3F6, 3E6, 2E7, and 10G3) protected Fisher 344 male rats after injected i.v. with mixtures of these MAb ascites and lethal toxin (Table 3). Lethal toxin [40 μg of PA and 8 μg of LF, approximately 13 LD_{50} (10)] was neutralized by 1 mg 3F6 or 5 mg of 3E6, 2E7, and 10G3 ascites Ig. A few Fisher 344 rats, however, did survive at 1 mg 2E7 (three out of seven) and 10G3 (two out of four) of ascites Ig. These rats appeared ill (lethargic, rapid respiration rate, and raised fur) for 4 to 5 h after toxin challenge before eventually recovering. Monoclone 3E6 did not appear to be able to neutralize lethal toxin as effectively as 3F6 at similar Ig concentrations. The difference may be attributed to Ig concentrations which were measured as total ascites Ig and not specific MAb Ig (5). Although 1D8 did not protect rats, there was a significant delay of time-to-death (mean time-to-death of 2.8 h at 5 mg Ig).

Incubation of the 63 MAbs at Ig concentrations up to 1 mg/ml with lethal toxin [400 ng PA + 40 ng LF; (approximately 27 times the concentration of LF that inhibited 50% of cell growth and viability, personal observations)] prior to exposure to cells demonstrated that neutralization was achieved with six different MAbs; 10G3 (3 μg ascites Ig), 2E7 (30 μg ascites Ig), 10D4, 10G4, and 13D10 (250 μg ascites Ig), and 1D8 (500 μg ascites Ig) (Fig. 2). We did not observe neutralization of lethal toxin in cell culture with either 3F6 or 3E6 at 1 mg ascites Ig. However,

three clones (10D4, 10G4, and 13D10) that did not protect rats against death with lethal toxin at the highest dose tested (1 mg Ig) or show an appreciable delay of time-to-death, neutralized lethal toxin in the cell culture assay. In addition, the clone that delayed time-to-death of rats (1D8) also neutralized lethal toxin in the cell culture assay. Identical results were obtained when MAbs were preincubated with LF (40 ng per ml) and then added to cells with bound PA or PA63 (400 ng per ml). Again, 3F6 and 3E6 did not neutralize lethal toxin. Only one of these MAbs (1D8) neutralized lethal toxin activity when added to cells to which LF was allowed to bind to PA by sequentially incubating J774A.1 cells with PA63 (800 ng per ml) then LF (80 ng per ml) at 4°C for 2 h each (Fig. 3).

Neutralization of lethal toxin was also demonstrated by the LDH assay in which J774A.1 cells, cultured in 24-well plates, were exposed to lethal toxin pretreated with MAbs. The percent neutralization at 100 μg MAb Ig, measured as a percent of control wells, indicated that 2E7 (102%), 10G3 (87%), 10G4 (51%), 10D4 (52%), 13D10 (51%), and 1D8 (89%) neutralized lethal toxin. Monoclonal antibodies 3F6 (5%) and 3E6 (6%) did not appear to neutralize lethal toxin in this assay. The percent neutralizing activity for the remaining MAbs ranged from 3.2 to 23%.

Competitive binding assay. The competitive ELISA was performed to determine whether the MAbs that neutralized lethal toxin, in either the in-vivo or in-vitro assays, were directed against overlapping or nonoverlapping antigenic determinants. Four

different competition groups could be identified on the basis of competitive binding experiments among the eight neutralizing MABs (Table 4). Similar antigenic regions were recognized by 3E6 and 3F6; 2E7 and 10G3; and 10G4, 10D4, and 13D10. Monoclone 1D8 reciprocally blocked only itself. We observed nonreciprocal or one-way inhibition by 10G4, 10D4, and 13D10, by blocking the binding of 2E7 and 10G3, both from the same determinant group. Monoclonal antibodies 3E6 and 3F6 enhanced binding of 2E7, 10G3, and 1D8, which suggested a conformational change in LF (27). Monoclonal antibody 1D8 also slightly enhanced the binding of 2E7 and 10G3. The low reciprocal percent values for 10G4 and 13D10 are reflected by their low affinity (Table 5 below). The antigenic determinant groups were further studied in a competition ELISA in which biotinylated 10G3 or 3F6 was tested against various concentrations of unlabeled, competing MAb (Fig. 4a and 4b). Monoclonal antibodies 10D4, 10G4, and 13D10 showed partial recognition with 10G3, especially at higher Ig concentrations (Fig. 4a). However, 3F6 (as well as 3E6) and 1D8 not only bound to a different antigenic site but again enhanced the A_{405} absorbance value of biotinylated 10G3. The initial binding of either 3F6 (or 3E6) or 1D8 to LF increased the apparent affinity of biotinylated 10G3 for LF. Similar results were observed with biotinylated 2E7 instead of 10G3. Data from the ELISA competition assay with biotinylated 3F6 (Fig. 4b; similar results were obtained with 3E6) demonstrated recognition of the same antigenic region by only 3F6.

Binding studies. Binding studies showed that the five MAbs that neutralized lethal toxin activity by interacting with LF in solution in the cellular assays (10G3, 2E7, 10D4, 10G4, and 13D10) inhibited binding of ^{125}I-labeled LF to PA bound to cell receptors from 70 to 97% (Table 5). Monoclonal antibody 1D8, which was able to neutralize lethal toxin activity in the cellular assays even after LF was prebound to cell-bound PA, inhibited the binding of ^{125}I-labeled LF to PA bound to cell receptors by 41%. The percent binding inhibition for both 3F6 and 3E6, which did not neutralize in the cellular assays, was 7%. Several other MAbs (3E3, 5E5, 5C8, 8F2, 9E5, and 9F10) blocked the binding of ^{125}I-LF to PA from 21 to 32% (Table 2). However, these MAbs did not appreciably delay the time-to-death of rats injected with lethal toxin and 1 mg of ascites Ig (range 1-1.75 h) and did not demonstrate neutralization in the macrophage cytolytic assays.

Table 5 summarizes the properties of these MAbs. The eight neutralizing MAbs also can be placed into one of three groups based upon the relative affinity measurements (31); high affinity ($\leq$ 0.2 μg), moderate affinity ($\geq$ 0.2 to $\leq$ 1 μg), and low affinity ($>$ 1 μg). Affinity measurements give a quantitative estimate of the antibody concentration required to achieve 50% plateau binding (29,30). We observed neutralization of lethal toxin by MAbs of moderate and low affinity.

DISCUSSION

Six MAbs (10G3, 2E7, 10G4, 10D4, 13D10, and 1D8) neutralized lethal toxin in both macrophage assays. Five of these MAbs (10G3, 2E7, 10G4, 10D4, and 13D10) appeared to do so by inhibiting the binding of LF to cell-bound PA. This was demonstrated directly by the ability of these MAbs to inhibit the binding of ^{125}I-LF to cell-bound PA and is supported by the results of the colorimetric assay, in which these MAbs were unable to protect cells to which PA and LF had been prebound. Among these MAbs there appeared to be a correlation between their ability to protect macrophages in-vitro and rats in-vivo. The MAbs that were most active in neutralizing toxin in-vitro had higher relative binding affinities and protected rats (10G3 and 2E7). Those MAbs which were less active in both in-vitro neutralization assays had lower binding affinities and did not protect rats (10G4, 10D4, and 13D10). It appears as if the in-vitro assays were more sensitive for these MAbs. This may be explained by the different ratios of MAb Ig:LF used in each assay. The ratio was 1 mg Ig:8 μg LF in the rat assay, 1 mg Ig:0.04 μg LF in the colorimetric assay, and 100 μg Ig:0.025 μg LF in the LDH assay. The 32- and 200-fold higher ratios of MAb Ig:LF in the LDH and colorimetric assays, respectively, may have enabled detection of neutralizing activity in those MAbs with lower binding affinity.

Monoclonal antibody 1D8 was unique in being the only MAb able to neutralize lethal toxin after PA and LF were

sequentially prebound to cells. We can only speculate that the binding site for 1D8 is different from the PA binding site on LF as 1D8 did not reciprocally compete in the competition ELISA with any of those MAbs that inhibited binding of ^{125}I-LF to PA and was unable to inhibit completely the binding of ^{125}I-LF to cell-bound PA.

We did not understand why MAb 3F6 was able to protect rats in-vivo but was unable to neutralize lethal toxin activity in the in-vitro macrophage assays. The inability of 3F6 to block LF binding to cell-bound PA suggests that it may be binding to the presumed enzymatically active region of LF. We were unable to evaluate the ability of 3F6 to neutralize enzymatic activity of LF since the nature of this activity is not yet known. However, it would be expected that such binding would be detected in the in-vitro neutralization assays if cytolysis is due to the postulated enzymatic activity of LF. It may be relevant that MAb and polyclonal antibodies to other toxins have been described that inhibit enzymatic activity but do not neutralize toxicity (7, 22). This may be explained by the removal of antibodies after endocytosis with restoration of toxic activity (7) which could occur in macrophages in-vitro but not in other target cells in the rat. This inability of 3F6 to neutralize toxin in-vitro also suggests that macrophage cytotoxicity may not be a direct correlate of lethality in the rat model. There may be other cell targets and mechanisms responsible for death in the rat. It is also possible that 3F6 when given to the rat, may direct the

toxin to other cells which are resistant to lethal toxin or it may effect the clearance of the lethal toxin in other unknown ways.

Competitive binding assays using MAbs have been useful in defining antigenic regions on proteins. This study identified four antigenic regions of the LF protein capable of inducing seven neutralizing MAbs. We observed reciprocal and nonreciprocal competition and synergistic, or enhanced binding, interactions. Nonreciprocal binding has been attributed to several factors, including conformational changes, steric hindrance, or avidity differences (1,6,14,23). Synergistic binding, as with 10G3, 2E7, and 1D8 in the presence of 3F6, suggests either an interaction among the Fc regions of the antibody molecules (9) or conformational changes of the antigen (27). Since the synergistic binding was nonreciprocal, the observed effect was probably due to a conformational change (27) in the LF protein.

Until recently very little homology was thought to exist between LF and EF proteins. However, a comparison of the deduced amino acid sequences of EF and LF suggest that there are several regions of homology in the N-termini of these proteins (4,25, J. R. Lowe, Ph.D. thesis, University of Kansas, Kansas City, 1989). We identified three LF MAbs that cross-reacted with EF by ELISA (10D4, 10G4, and 10B2). Two of these MAbs (10D4 and 10G4), which blocked the binding of LF to PA, may recognize antigenic determinants within these regions of homology. There

was no inhibition of LF binding with MAb 10B2, suggesting that LF and EF may also share a determinant that does not involve a LF binding site to cell-bound PA. The two MAbs that best blocked binding of LF to PA (2E7 and 10G3) and were assumed to be reacting at a binding site, did not cross-react with EF.

It is anticipated that our MAbs should prove useful for studies of antigenic structure and function of LF, interactions of LF with PA, and possible relationships between LF and EF.

ACKNOWLEDGMENTS

We thank C. Osterling for preparation and maintenance of the hybridomas and B. Barrido, S. Tobery, and P. Merrill for technical assistance.

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TABLE 1. Immunization of female BALB/c mice with purified LF Antigen

Fusion	Immunization route	LF (μg)	Adjuvant (0.5 ml)[a]	Injection schedule
I & II	im	12 μg	FCA	Day 0
	im	12 μg	FCA	2 weeks
	im	25 μg	FCA	8 weeks
	im	25 μg	FIA	12 weeks
	iv	25 μg	none	30 weeks[b]
III	im	1 μg	FCA	0 weeks
	im	10 μg	FIA	3 weeks
	im	10 μg	FIA	7 weeks
	iv	50 μg	none	14 weeks[b]

[a] FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant.

[b] Fusions were done 3 days after the last immunization.

TABLE 2. Hybridoma clones producing MAb against LF obtained in three separate fusions along with their subtypes and percent inhibition of binding ^{125}I-LF to PA[a]

Fusion	Hybridoma Clone	MAb Subtype	Binding Inhibition[b]
LF I	1D8-1-1	G_1	41
	2E7-1-1	G_1	97
	3E6-1-1	G_1	7
	3F6-1-1	G_1	7
LF II	1F11-2-1[c]	G_1	15
	2G2-1-1[c]	M	6[e]
	3E3-1-1	M	7
	5E5-1-1	G_1	32
LF III	5C8-1-2	G_1	21
	8F2-2-2	G_1	22
	9E5-1-1	G_1	32
	9F10-1-1	G_1	32
	10B2-2-1[d]	M	-5
	10D4-1-1[d]	G_3	88
	10G3-2-1	G_1	95
	10G4-1-1[d]	G_3	77
	13D10-1-1	G_1	70

[a] Table 2 lists only those clones mentioned in the text.

[b] Percent inhibition of binding of ^{125}I-LF to PA.

[c] Reacts to LF and PA by ELISA.

[d] Reacts to LF and EF by ELISA.

[e] Tested at 20 μg Ig.

TABLE 3. Percent survival of Fisher 344 rats injected with lethal toxin and MAb Ig

Monoclone	1 mg Ig			5 mg Ig		
	Survivors / Total	%	MTTD[a]	Survivors / Total	%	MTTD
3F6	5/5	100	-	2/2	100	-
3E6	0/2	0	2.1 ± 1.1	3/3	100	-
2E7	3/7	43	10.5 ± 8.5	8/8	100	-
10G3	2/4	50	5.8 ± 0.9	4/4	100	-
1D8	0/2	0	2.0 ± 0.1	0/4	0	2.8 ± 0.1

[a] MTTD, Mean time-to-death (h). The MTTD for the other 59 monoclones ranged from 0.9 to 1.75 h. Control rats averaged 1.1 ± 0.1 h mean time-to-death.

TABLE 4. Competitive antibody-binding ELISA between selected biotinylated and unlabeled neutralizing MAbs[a]

Unlabeled MAb (MAb_1)	Biotinylated MAb (MAb_2)							
	3E6	3F6	2E7	10G3	10G4	10D4	13D10	1D8
3E6	14	7	294	211	90	97	84	218
3F6	4	4	408	208	81	100	62	209
2E7	71	63	7	0	60	54	42	96
10G3	43	40	4	0	29	42	32	67
10G4	82	95	8	18	58	46	27	71
10D4	62	76	1	9	37	21	31	58
13D10	73	88	11	21	34	33	32	41
1D8	38	40	145	143	33	23	18	1

[a] Number values indicate the percent of biotin-labeled MAb bound in the presence of a saturating concentration of a second MAb. Determinant groups are identified by boxes.

TABLE 5. Summary of properties of neutralizing LF MAbs

Hybridoma Clone	Antibody Subtype	Neutralization			Binding Inhibition[d]	Affinity (μg)[e]
		Rats[a]	MTT[b]	LDH[c]		
2E7	IgG_1	5	30	102	97	1.3
3E6	IgG_1	5	>1000	7	7	0.2
3F6	IgG_1	1	>1000	3	7	0.6
10G3	IgG_1	5	3	87	95	0.3
10D4	IgG_3	neg[f]	250	52	88	7.9
10G4	IgG_3	neg	250	51	77	4.6
13D10	IgG_1	neg	250	51	70	7.8
1D8	IgG_1	>5	500	89	41	0.2

[a] Amount of Ig (mg) required to neutralize lethal toxin in the rat (see Table 3).

[b] Amount of Ig (μg) required to neutralize lethal toxin measured by the reduction of MTT (colorimetric macrophage assay) (see Fig. 3).

[c] Percent neutralization of lethal toxin in the LDH assay (see text).

[d] Percent inhibition of binding of ^{125}I-LF to PA bound on L6 cells (from Table 2).

[e] Amount of Ig (μg) required for 50% of plateau binding by ELISA.

[f] neg, negative.

FIGURE LEGENDS

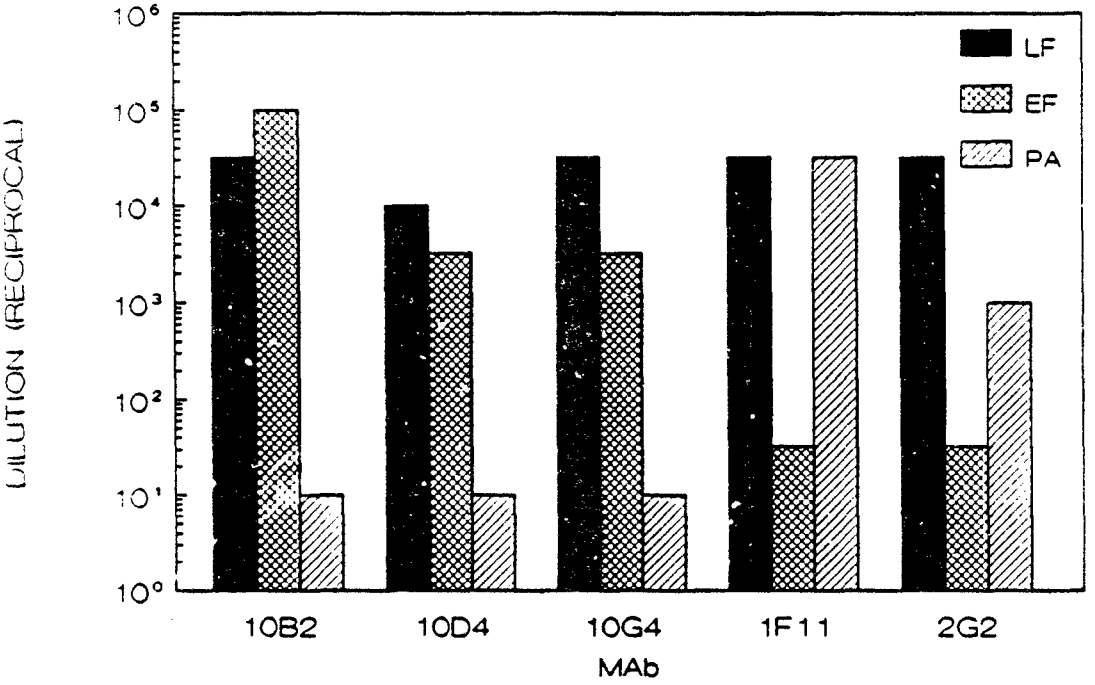
FIGURE 1. ELISA titers of LF MAbs that reacted with EF and PA antigens.

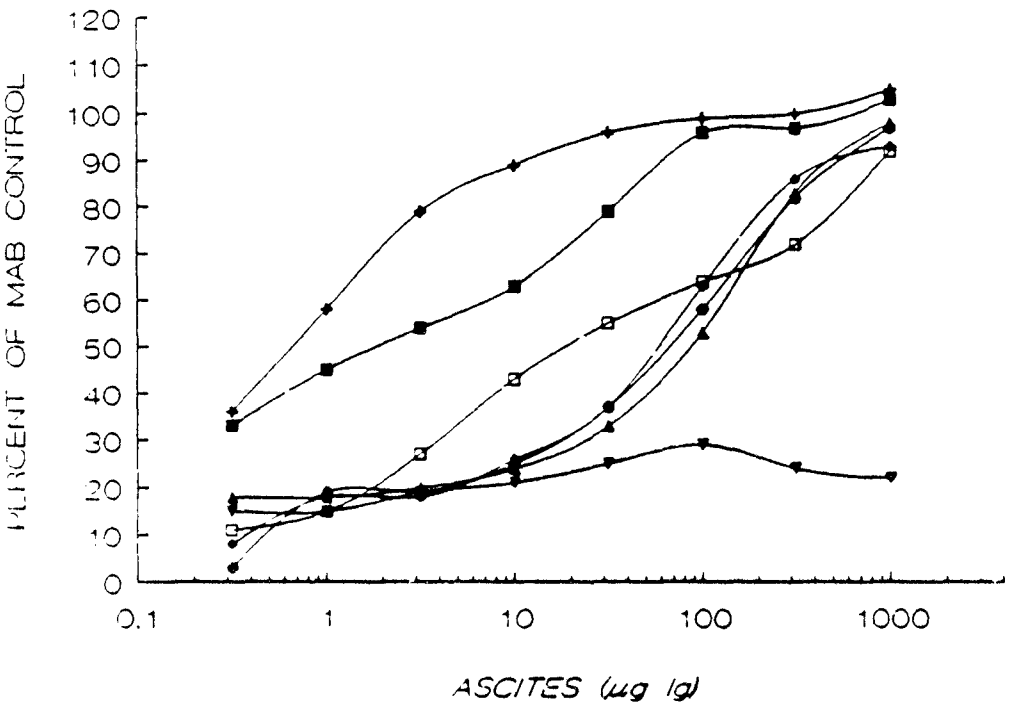
FIGURE 2. Ability of LF MAbs to neutralize lethal toxin activity. Colorimetric assay used to calculate the percent of J774A.1 cells surviving in the presence of lethal toxin (400 ng PA per ml and 40 ng LF per ml) preincubated with MAbs before adding to cells: ✚ 10G3, ■ 2E7, ◆ 10D4, ▲ 10G4, ● 13D10, ▼ 3F6, □ 1D8.

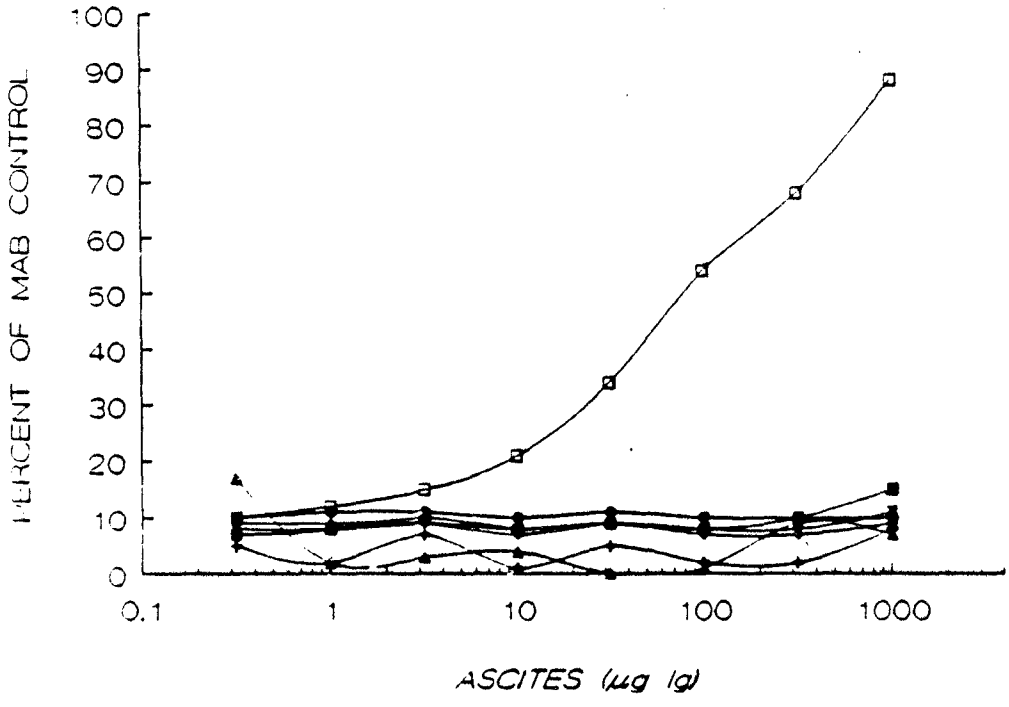
FIGURE 3. Ability of LF MAbs to neutralize lethal toxin activity. Colorimetric assay used to calculate the percent of J774A.1 cells surviving after sequentially preincubating cells with PA63 (800 ng per ml) and LF (80 ng per ml) followed by addition of MAbs: ✚ 10G3, ■ 2E7, ◆ 10D4, ▲ 10G4, ● 13D10, ▼ 3F6, □ 1D8.

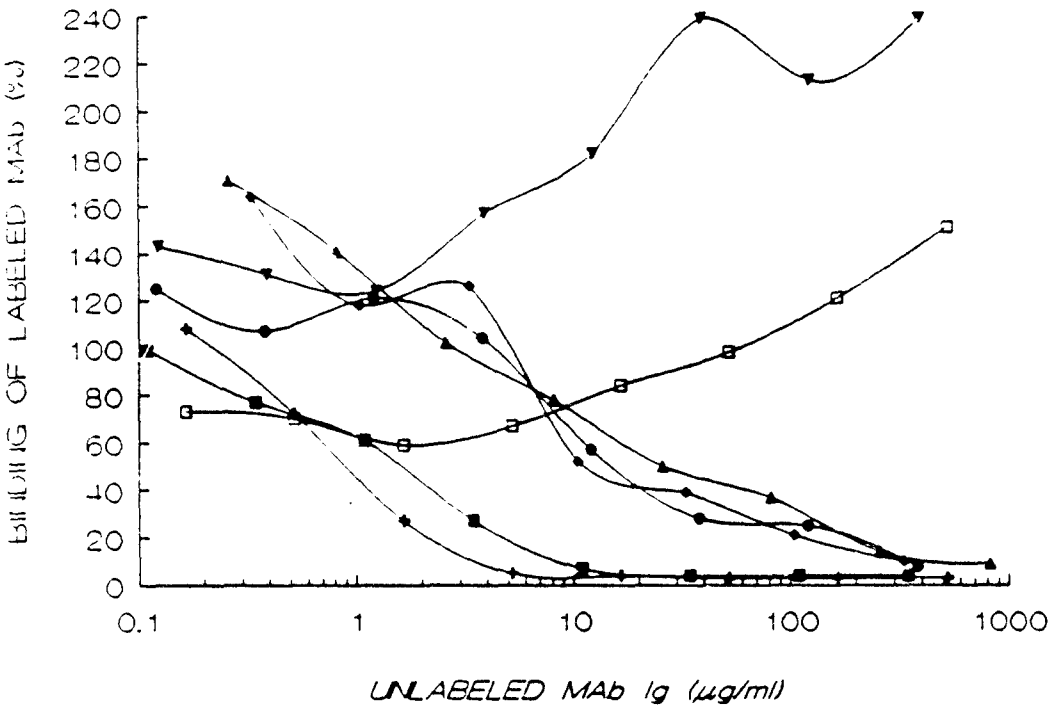
FIGURE 4a. Competitive antibody-binding assay with biotinylated and unlabeled MAbs. The binding of biotinylated 10G3 was measured in the presence of different concentrations of competing, unlabeled MAbs: ✚ 10G3, ■ 2E7, ◆ 10D4, ▲ 10G4, ● 13D10, ▼ 3F6, □ 1D8.

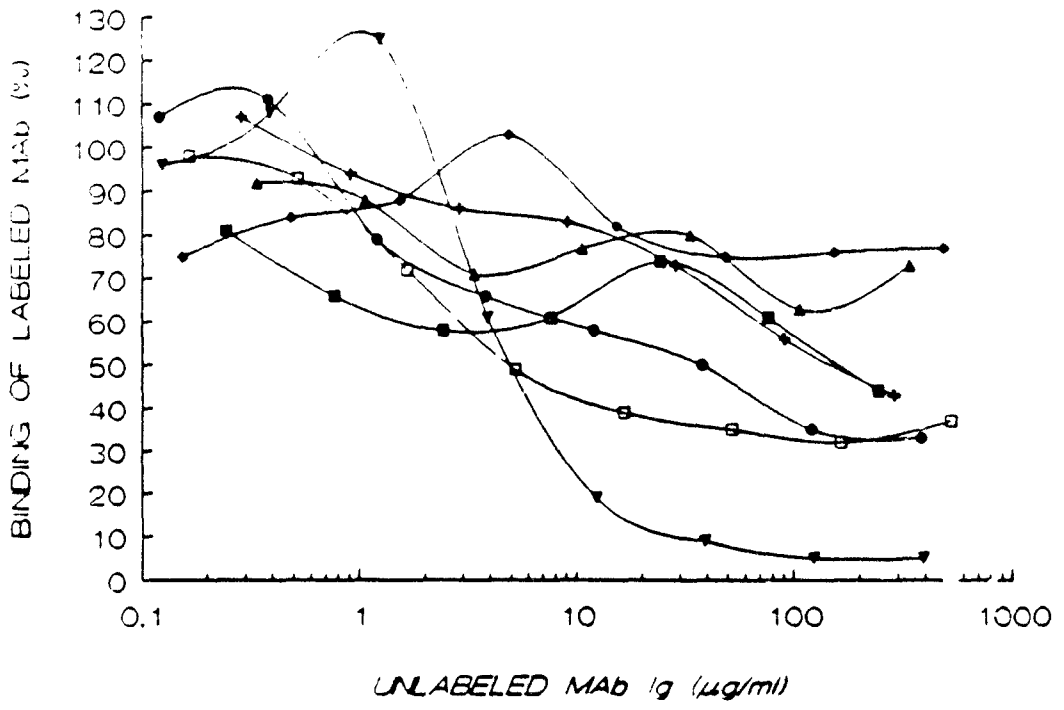
FIGURE 4b. Competitive antibody-binding assay with biotinylated and unlabeled MAbs. The binding of biotinylated 3F6 was measured in the presence of different concentrations of competing, unlabeled MAbs: ✚ 10G3, ■ 2E7, ◆ 10D4, ▲ 10G4, ● 13D10, ▼ 3F6, □ 1D8.


DILUTION (RECIPROCAL)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
LF
EF
PA
10B2
10D4
10G4
1F11
2G2
MAb


PERCENT OF MAB CONTROL
120
110
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
100
1000
ASCITES (µg /g)


PERCENT OF MAB CONTROL
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
100
1000
ASCITES (µg /g)


BINDING OF LABELED MAb (%)
240
220
200
180
160
140
120
100
80
60
40
20
0
0.1
1
10
100
1000
UNLABELED MAb Ig (μg/ml)


BINDING OF LABELED MAb (%)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0.1
1
10
100
1000
UNLABELED MAb Ig (μg/ml)